VALIDATION OF A RECURRENT NITROGLYCERIN MIGRAINE MODEL IN THE RAT

by
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ABSTRACT
AINSLEE PATRICIA JOHNSON: Validation of a Recurrent Nitroglycerin Migraine Model in the Rat
(Under the direction of Kenneth Sufka)

The present research sought to determine if repeated nitroglycerin (NTG) administrations elicited responses on clinically relevant behavioral endpoints of migraine in rats. Rats were given five saline, three NTG or five NTG administrations over a two-week period. After their fifth injection rats were evaluated using the rat grimace scale, modified light/dark box, thermal tail flick tests, elevated plus maze and forced swim test. Repeated NTG administration affected weight gain, movement in the modified light/dark box and photophobia. The thermal tail flick tests, elevated plus maze and forced swim test did not reveal treatment effects. Based on these findings, we conclude that the recurrent NTG migraine model is a valid and clinically relevant simulation of human migraine.

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1. Introduction

Migraine is a common headache disorder that causes significant disability for 13% of the United States population and 11% of people worldwide (Lipton, S. Diamond, Reed, M. Diamond & Stewart, 2007; Stovner et al., 2007). Migraine is more prevalent in women than men, affecting 18% of women and 6% of men (Lipton, Bigal & Diamond, 2007). Migraine suffering is not only extremely common, but also substantially debilitating. The World Health Organization ranks migraine among the diseases that cause significant disability (World Health Organization [WHO], 2012).

A clinical migraine diagnosis requires the presence of specific criteria that range in frequency and severity. The first requirement is a frequency of at least 5 recurrent migraine episodes. Migraine episodes are characterized by an intense headache that lasts 4-72 hours if left untreated (International Headache Society [IHS], 2005). Migraine attacks may or may not be preceded by unusual sensory or visual sensations known as aura. The headache pain associated with migraine typically has a throbbing quality and unilateral location that may be aggravated by physical activity (IHS, 2005). Common symptoms of migraine include nausea, vomiting, and an increased sensitivity to light and sound, known as photophobia and phonophobia respectively (IHS, 2005)

Migraine episodes may become more frequent and intense over time, and eventually transform into chronic migraine. A chronic migraine diagnosis requires 15 or more headache days per month for at least 3 months (IHS, 2005). These recurring

migraine attacks routinely disturb the individual's quality of life, daily activities, and even their financial state (WHO, 2012). The progression of migraine to chronic migraine is associated with the development of allodynia, or a pain response to non-noxious stimulus (Bigal, Ferrari, Silberstein, Lipton & Goadsby, 2009). Cutaneous allodynia is the type most commonly associated with migraine. This causes normally painless stimuli to the head region to become a painful experience for the migraineur.

Another characteristic that many migraine sufferers share is the co-occurrence of psychiatric disorders such as anxiety and depression. Several studies have found that migraine sufferers are 2-4 times more likely display anxiety and depression than non-migraine sufferers (Breslau, Davis, Schultz, & Peterson, 1994; Lantéri-Minet, Radat, Chautart & Lucas 2005; Jette, Patten, Williams, Becke & Wiebe, 2008). There is also a strong relationship between the frequency of migraine attacks and the odds of displaying psychiatric comorbidity (Zwart, Dyb, Hagen, Ødegård… & Stovner, 2003). A cross-sectional population study found that chronic migraineurs were 6-7 times more likely to express comorbid anxiety and depression than headache-free individuals (Zwart et al., 2003). Individuals experiencing less frequent attacks, on the other hand, were twice less likely to present these conditions, indicating a higher prevalence of psychiatric comorbidity in migraineurs with more frequent attacks (Zwart et al., 2003). When rates of comorbid anxiety and depression are examined separately, anxiety consistently shows higher associations with migraine than depression does (Zwart et al., 2003; Merikangas, Angst & Isler, 1990).

Migraine suffering impairs individuals both physically and psychologically. As a result, two classes of drug treatments have been developed: acute and preventative

migraine treatments. Several acute treatments are currently used to reverse migraine pain once an attack has already started. These include simple analgesics, NSAIDs, opioids, ergots and triptans. The most common acute treatments are ergots and triptans. Both ergots and triptans alter blood flow in the brain, which led to the belief that the mechanism behind migraine was purely vascular (Humphrey, Feniuk, Perren, Beresford… & Whalley, 1990). Triptans seem to be more efficacious than ergots because they not only relieve headache, but also reduce the nausea and vomiting associated with migraine attacks (Silberstein, 2004). While the enhanced therapeutic effect of triptans has led researchers to re-examine the pathophysiology of migraine, their efficacy is still limited (Humphrey et al., 1990). A comprehensive meta-analysis of sumatriptan, the gold standard migraine treatment, revealed a disappointing response rate of 59% (Ferrari, Roon, Lipton, & Goadsby, 2001).

While acute migraine treatments are somewhat effective in reducing migraine pain, they do not reduce the likelihood of attacks occurring in the future (Silberstein, 2004). Their therapeutic effect is also limited to administration early on in the progression of the attack (Silberstein, 2004). This is problematic for patients without aura because catching the attack prior to onset is not always possible. The overuse of acute medications to alleviate migraine pain is also a common problem because it can lead to a considerable increase in the frequency of migraine attacks (Katsarava, Limmroth, Finke, Diener & Fritsche, 2003).

The preventative, or prophylactic, class of migraine treatments are designed to reduce the likelihood of migraine attacks occurring in the future. Only 13% of migraine sufferers currently use any type of prophylactic treatment (Lipton, Bigal & Diamond,

2007). Some of the most familiar prophylactic treatments include beta-blockers, antidepressants, and anticonvulsants (Silberstein, 2004). These treatments are moderately effective for many, but they tend to have considerable adverse side effects such as weight gain, fatigue, drowsiness, and nausea (Silberstein, 2004).

Despite the striking prevalence and burden of migraine suffering, treatments available for this condition are surprisingly limited. The paucity of clinically effective anti-migraine treatments that are void of serious side effects may reflect the absence of a valid animal simulation that could be useful for preclinical drug treatment screenings. An animal model that effectively characterizes migraine behavior is necessary to better understand and treat migraine.

When animal models are developed and utilized correctly, they expand upon the existing knowledge of a clinical syndrome. A clinically relevant animal model demonstrates homologies, or behavioral similarities, between the human clinical profile and animals' behavioral responses (Willner, 1991). This concept, known as endophenotypic mapping, provides animal models with their clinical efficacy and validity (Willner, 1991). Endophenotypic mapping allows for the generalizablility of a clinical syndrome across species, making it possible to collect preclinical data without introducing any risks to human populations (van der Staay, 2006). The development of a clinically relevant migraine model is essential to improve both the treatment methods and the understanding of migraine pathophysiology.

The finding that nitroglycerin (NTG) induces migraine in human migraineurs sparked the discussion of an NTG rodent model of migraine (Iversen, 2001). The existing NTG migraine literature has examined the effects of NTG on two endophenotypes:

hyperalgesia and allodynia.

One study explored NTG-induced hyperalgesia in rats measured by a tail flick test and found that NTG induced hyperalgesia 2 and 4 hours post NTG administration (Costa, Smeraldi, Tassorelli, Greco & Nappi, 2005). Another group examined the effect of both acute and chronic NTG administration on mechanical hyperalgesia in mice, using the von Frey method. These researchers also found that acute and chronic NTG exposure induced mechanical hyperalgesia (Pradhan et al., 2013).

The dependent measure of these studies is hyperalgesia, which is a heightened pain response to an already painful stimulus. Allodynia, or the development of a pain response to a non-painful stimulus, is sometimes seen in chronic migraineurs (Bigal et al., 2009). Hyperalgesia, however, is not. While hyperalgesia and allodynia are related, they are not equivalent and may act on different nociceptive pathways in the brain. Because hyperalgesia merely indicates the presence of nonspecific pain and is not commonly seen in human migraine, it is a clinically irrelevant behavioral endpoint for this condition.

One study has examined the effect of NTG on the more clinically relevant endpoint of allodynia in mice (Bates et al., 2010). Animals were given a single NTG administration at various doses, followed by either sumatriptan or saline. Thermal allodynia was assessed via the Hargreaves assay and mechanical allodynia was measured using the von Frey apparatus. The highest doses of NTG produced both thermal and mechanical hyperalgesia that were effectively reversed by sumatriptan at 90 and 120 minutes (Bates et al., 2010).

While allodynia is more clinically relevant than hyperalgesia, it is rarely seen in migraineurs who experience less frequent headaches. Additionally, the Bates et al. study

examined the effects of a single NTG exposure, which does not correspond to the attack frequency of human migraineurs who typically experience allodynia (Bigal et al., 2009). For these reasons, additional homologies must be established through endophenotypic mapping to truly validate the NTG migraine model in rodents.

Previous work from this laboratory has examined the effects of a single NTG-induced migraine episode on more clinically relevant behavioral endpoints, such as (a) pain; (b) photophobia; (c) sensitivity to movement and (d) thermal allodynia. Two hours after rats received a single 10 mg/kg dose of NTG, pain was quantified using the rat grimace scale and both photophobia and movement were measured using a traditional light/dark box and a modified light/dark box. Movement was additionally assessed using the rat Rotor-Rod and thermal allodynia was evaluated using a hot and cold tail flick test. Allodynia was measured in an attempt to replicate the findings of the Bates et al. study. Rat grimace scale scores approached significance, with treatment animals obtaining higher pain scores than control animals. The presence of photophobia was not detected by any of the aforementioned measures. Movement in the modified light/dark box was significant, but contrary to our predictions, treatment animals attained higher movement scores than control animals. The results of the Rotor-Rod indicated the same trend for movement; treatment animals attained a longer latency to fall, and thus moved more, than control animals. The difference between groups for movement on the Rotor-Rod approached significance.

A possible explanation for the discrepancy between these results and our predictions is that animals were tested during their first, and only, NTG migraine episode. Because the migraine episode was a novel experience, animals may have attempted to

escape or avoid migraine pain by moving around. The Rotor-Rod also presents the confounding variable of fear, as falling off the Rotor-Rod is a painful experience and may have heightened the animals' anxiety. Avoidance behavior and the fear of falling off the Rotor-Rod likely caused treatment animals to move more than control animals, in contrast to our predictions. Another problem with one migraine episode is that human clinical diagnosis requires a minimum of 5 migraine attacks. From a clinical standpoint, one migraine episode does not simulate the human migraine condition and may, therefore, fail to produce the symptoms expected to accompany migraine episodes in rats.

To correct these methodological issues in the present study, we developed two recurring migraine conditions. Animals received 5 saline, 3 NTG or 5 NTG administrations. The Rotor-Rod was abandoned and movement and photophobia were simultaneously assessed using the modified light/dark box. Tests were conducted two hours after the animals' last NTG administration and the following day. The goal of the present study was twofold. The first was to determine whether or not the clinically relevant behavioral end points previously explored (pain, photophobia, movement and thermal allodynia) were present under the new, recurring migraine condition. We predicted that animals receiving either 3 or 5 NTG migraine episodes would obtain higher pain scores on the rat grimace scale, spend less time in the light chamber of a modified light/dark box, display reduced movement, and exhibit a shorter latency to tail flick on the thermal tail flick tests than animals experiencing no NTG migraine episodes.

The second objective of the present study was to determine whether or not animals experiencing multiple migraine episodes displayed the comorbidity of anxiety and depression experienced by many human migraineurs, and most commonly seen in the

chronic migraine subpopulation. This was determined using two empirically valid measures of anxiety and depression in rodents: the elevated plus maze and the forced swim test. We predicted that animals receiving 3 or 5 NTG migraine episodes would enter the open arms less often and spend less time in the open arms than control animals. We also predicted that treatment animals would obtain higher float times than control animals at both the 5 and 10-minute intervals of the forced swim test.

2. Materials and Methods

2.1. Animals

Experiments were performed on 30 adult male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) weighing between 225 and 320 g. Rats were housed two per cage at 22°C on a 12-hour light/dark cycle. The base of each cage was clear and plastic (Ancare), measuring 22.4 x 21.0 x 13.3 cm. Food (Teklad 7001, Teklad Diets, Madison, WI, USA) and water were available *ad libitum* via a wire lid on top of the cage. Each cage was given a single circular enclosure made of PVC pipe. The enclosure measured 16.5 cm in diameter by 7.5 cm tall, with an arched opening. The coverings were included to allow the rats to avoid light during the NTG migraine episodes in their home cages. All procedures were approved by the University of Mississippi IACUC (protocol number 13-023).

2.2. Design

This study employed a three-group design in which 10 rats were randomly assigned one of three experimental conditions. Rats received a series of 5 intraperitoneal (IP) injections over the course of two weeks. The control group received 5 saline administrations, the second group received 3 NTG alternated with 2 saline administrations and the third group received 5 NTG administrations. The NTG (Copperhead Chemical Company Inc., SDM®27, Tamaqua, PA, USA) concentration was

5 mg/kg dissolved in 50% ethanol and 50% propylene glycol. NTG was administered in a volume of 2 ml/kg for a final dose of 10 mg/kg. Physiological saline was used for the control group. Rats were divided into two, roughly equal squads ($s_1 = 14$; $s_2 = 16$) to accommodate testing. Squads were injected and tested two days apart from each other.

2.3. Procedure

Two hours after the 5th NTG or saline administration, rats were taken to the procedure rooms and run through a series of assays. These included a picture of the rat's face to quantify the rat grimace scale, a modified light/dark box test and two nociceptive tests. The following day, rats were tested on the elevated plus maze in the morning and the forced swim test in the afternoon. A description of these assays is detailed below.

2.3.1. Rat grimace scale

The rat grimace scale (RGS) is designed to quantify a rat's facial expression related to pain (Sotocinal et al., 2011). Two hours after their last NTG or saline administration, rats were placed in a 31.1 cm x 21.6 x 26.0 cm clear Plexiglas chamber with a wire bottom. Pictures were taken of their face, ears and whiskers using a Nikon 16.1 megapixel camera. A dry-erase board was placed behind the chamber to identify the subject number. After each picture, the apparatus was wiped down with a Clorox wipe before proceeding with the next test. Scorers were trained using the operational definitions and examples from the work of the Sotocinal et al. study. The pictures were evaluated for four facial characteristics: orbital tightening, nose or cheek flattening, ears pointing outwards, and changes in whisker position, such as whiskers moving forward or

closer together (Sotocinal et al., 2011). Each of these facial characteristics was scored with either a 0, indicating a high confidence that the characteristic was absent, 1, indicating either a high confidence that the characteristic was moderately present or that the rater was unsure about the presence of the characteristic, or a 2, indicating a high confidence that the characteristic was present (Sotocinal et al., 2011). Scores from two raters were averaged to produce an overall pain score for each rat. The percent agreement between two raters was 84%.

2.3.2. Modified light/dark box

Migraine episodes are associated with decreased motor activity and the avoidance of well-lit areas. To quantify both measures we used a conditioned place preference apparatus (Model # ENV-013, Med Associates Inc., St. Albans, VT, USA). For this experiment, the tops of black and grey chambers, which are usually clear, were covered with black poster board. The top of white chamber remained uncovered to create a light and dark environment in the same apparatus. Following their RGS picture, rats were transferred directly from the RGS box to the grey chamber of the modified light/dark box. They were left in the grey chamber with the guillotine doors down for 1 minute to acclimate. After the acclimation period, the doors were opened simultaneously and animals were free to explore the apparatus. During the 10-minute test session, the amount of time spent in each chamber and the number of photo beam breaks were recorded. After each test session, the apparatus was wiped down with a Clorox wipe before proceeding with the next test.

2.3.3. Thermal tail flick test

Upon removal from modified light/dark box, animals were administered two nociceptive tests to quantify allodynia associated with NTG administration. One day prior to the test session, rats' tails were marked 5 cm from the tip and they were gently restrained with a towel for 40 seconds to become acclimated with the testing procedure. Two water baths were maintained in glass cylinders at 46°C and 15°C using a hot plate (Harvard) and ice respectively. These temperatures were not inherently painful, thereby measuring thermal allodynia as opposed to hyperalgesia. On test day, rats were wrapped in a towel and their tails were immersed to the 5 cm mark in the hot or cold water bath. The latency to tail flick was recorded. A tail flick was defined as a pronounced flick or curl out of the water. The maximum length of the test session was 40 seconds. Tests were counterbalanced for order and rats were returned to their home cages following this test.

2.3.4. Elevated plus maze

The morning following the last NTG administration, rats were tested on the elevated plus maze to quantify anxiety-like behavior. The maze is a plus-shaped apparatus with four arms measuring 56 cm in length and elevated 76 cm above the ground. The perpendicular, "closed" arms of the maze have 19 cm high sidewalls, while the perpendicular, "open" arms lack sidewalls and resemble an open runway. Rats were transferred from their home cage directly to the procedure room and placed in the center of the maze, facing an open arm. The test session lasted 5 minutes and two dependent measures were recorded: the number of entries into the open arms and the time spent in the open arms. An entry into an open arm was defined as all four paws crossing the center

of the maze, where the open and closed arms meet, and into an open arm. After each test session, the apparatus was wiped down with a Clorox wipe and the animals were returned to their home cages.

2.3.5. Forced swim test

Four hours after testing in the elevated plus maze, rats were given a forced swim test to quantify depression or behavioral despair. Two plastic cylinders, measuring 20 cm in diameter and 35 cm in height, were separated by an opaque divider and filled to a height of 30 cm with room temperature water (24-26°C). Rats from the same home cage were simultaneously placed in the cylinders and observed for 10 minutes. Floating behavior was defined as small, involuntary movements that were necessary for the rat to keep their head above water. Float time was recorded at the 5-minute interval and when the 10-minute test session ended. Any attempts to escape, including vigorous swimming and submerging to explore the cylinder, were considered struggling behavior and were not included in the float time measure. After the test session ended, rats were removed from the apparatus and dried with microfiber towels before returning to their home cage.

2.4. Statistical analyses

All dependent measures were analyzed using a repeated measures ANOVA or a one-way ANOVA via the SPSS software. Post hoc tests were conducted using Fisher's LSD. Statistical significance was set at $p < .05$.

3. Results

The effects of NTG administration on percent weight gain are summarized in Figure 1, panels A and B. Figure 1A displays the percent weight change over the three day period following each treatment day. The highest percent weight gain was seen after saline administrations. The lowest percent weight gain was seen after NTG administrations. This trend was most pronounced in the 3 NTG group, which received NTG on trial days one and three and received saline on trial days two and four. A repeated measures ANOVA of these data revealed a significant treatment effect, $F(2,27) = 6.361$, $p = .005$. Post hoc analyses revealed that three days after their first trial, the 3 and 5 NTG groups gained significantly less weight than the control group ($p = .029$). Three days after their second trial, the 5 NTG group gained significantly less weight than the control group ($p = .006$). There were no significant group differences for percent weight change after the third trial. Three days after the fourth trial, however, the 3 NTG group gained significantly more weight than the saline group ($p = .013$).

Figure 1B displays the effects of NTG administration on the overall percent weight gain during the two-week injection period. The control group gained an average of 22% of their original weight while treatment groups gained approximately 14%. A one-way ANOVA of these data revealed a significant treatment effect, $F(2,29) = 6.929$, $p = .004$. Post hoc analyses revealed that both the 3 and 5 NTG groups gained significantly less weight than the control group over the two-week injection period (ps = .005 - .021).

The effects of NTG administration on rat grimace scale pain scores are summarized in Figure 2. The scores of two raters were averaged to obtain an overall pain score for each animal. The control group scored .075, while the 3 NTG group scored .14 and the 5 NTG group scored .20. A one-way ANOVA of these data failed to reveal a significant treatment effect, p = n.s. No further analyses were conducted on these data.

The effects of NTG administration on movement are summarized in Figure 3. The average movement score for the control group was 332 while that of the treatment groups was approximately 212. A one-way ANOVA of these data revealed a significant treatment effect, $F(2,29) = 4.529$, $p = .02$. Post hoc analyses revealed that both the 3 and 5 NTG groups had significantly lower movement scores than the control group (ps = .036 - .04).

The effects of NTG administration on the modified light/dark box are summarized in Figure 4. Control animals spent 61 seconds in the light chamber of the apparatus while the 3 NTG group spent 34 seconds and the 5 NTG group spent 14 seconds. A one-way ANOVA of these data revealed a significant treatment effect, $F(2,29) = 5.97$, $p = .007$. Post hoc analyses revealed that the difference seen in the 3 NTG approached significance when compared to the control group ($p = .055$) and the 5 NTG group spent significantly less time in the light chamber than the control group ($p = .005$).

The results of the thermal tail flick tests are summarized in Figures 5 and 6. Figure 5 displays the effect of NTG administration on the hot tail flick test. The mean latency to tail flick of the control group was 9.8 seconds while that of the 3 NTG group was 10.4 seconds and that of 5 NTG group was 13.2 seconds. A one-way ANOVA of these data failed to reveal a significant treatment effect, p = n.s. No further analyses were

conducted on these data. Figure 6 displays the effect of NTG administration on the cold tail flick test. The mean latency to tail flick for the control group was 16.5 seconds while that of the 3 NTG group was 23 seconds and that of the 5 NTG group was 20.8 seconds. A one-way ANOVA of these data failed to reveal a significant treatment effect, p = n.s. No further analyses were conducted on these data.

The results of the elevated plus maze are summarized in Figures 7 and 8. Figure 7 displays the effect of NTG administration on the number of open arm entries. The control group averaged 1.5 entries into the open arms while the 3 NTG group averaged 1.6 and the 5 NTG group averaged 1.4 entries. A one-way ANOVA of these data failed to reveal a significant treatment effect, p = n.s. No further analyses were conducted on these data. Figure 8 displays the effect of NTG on the time spent in the open arms of the maze. The control group spent an average of 13 seconds in the open arms while the 3 NTG group spent 17.4 seconds and the 5 NTG group spent 16.2 seconds. A one-way ANOVA of these data failed to reveal a significant treatment effect, p = n.s. No further analyses were conducted on these data.

The results of the forced swim test are summarized in Figures 9 and 10. Figure 9 displays the effect of NTG administration on float time at 5 minutes. The control group floated for an average of 136 seconds while the 3 NTG group floated for 145 seconds and the 5 NTG group floated for 113 seconds. A one-way ANOVA of these data failed to reveal a significant treatment effect, p = n.s. No further analyses were conducted on these data. Figure 10 displays the effect of NTG administration on float time at 10 minutes. The control group floated for an average of 420 seconds while the 3 NTG group floated for 424 seconds and the 5 NTG group floated for 362 seconds. A one-way ANOVA of

these data failed to reveal a significant treatment effect, p = n.s. No further analyses were conducted on these data.

4. Discussion

The goal of this study was to expand upon the rodent nitroglycerin (NTG) model of migraine to include a recurring migraine condition under which the behavioral endpoints of pain, photophobia, movement, allodynia and comorbid psychiatric disorders were evaluated. Rats were given 5 IP injections over the course of two weeks. One group received physiological saline for each injection, the second group received 3 NTG alternated with 2 saline injections, and the third group received NTG for each injection. Two hours after their last NTG or saline administration, animals were run through a variety of assays including a picture to quantify pain on the rat grimace scale, a modified light/dark box to measure photophobia and movement, and two tail flick tests to quantify thermal allodynia. The following day, animals were tested on the elevated plus maze to assess anxiety and the forced swim test to assess depression or behavioral despair.

An unexpected finding of the present research that parallels the clinical picture, and may provide additional support for this model, was the percent weight change among groups. Common features of human migraine episodes include nausea and vomiting. When people feel nauseous they eat less, and consistent appetite suppression may be manifested by changes in body weight. Therefore, changes in body weight may be an indirect measure of nausea, or at the very least, eating behavior in rats. Our animals gained less weight following treatment days involving NTG administrations than saline administrations, and these findings were consistent across groups. Treatment animals also

gained less weight overall during the two-week injection period than control animals. These findings could indicate that NTG administration caused nausea, which decreased animals' appetites and consequently impeded their weight gain. This interpretation of NTG-induced changes in body weight provides an unexpected behavioral endpoint that may add to the validity of this model.

Obesity is a risk factor for migraine in human populations, but the elevated percent weight gain of the control group compared to treatment groups should not be interpreted as an indication of obesity. Animals gain weight consistently in the lab because they are still growing, so this measure simply compares the expected weight gain seen in the control group to the unusual patterns of weight gain in the treatment groups.

The defining feature of human migraine is moderate to severe headache pain. In order to assess pain in this study, animals were photographed two hours post NTG administration and rated using the rat grimace scale (RGS). The 5 NTG group obtained higher overall pain scores than the 3 NTG group, who in turn, scored higher than the saline group, following our predictions. These findings, while modest, suggest that NTG animals were in a higher pain state than control animals.

It is not surprising that these results were not statistically significant due to the limited scoring of this scale (0,1, or 2). The findings of the present study align with the previous work from this lab, with similar overall pain scores for treatment and control animals at the two-hour time point. The RGS scores from the present study do not approach that of the original RGS research, but this was expected because the pain assays used in the Sotocinal et al. study appear to be more invasive and severe that of an NTG migraine episode (Sotocinal et al., 2011).

There is no extant data of RGS scores in the NTG migraine model, but the fact that our scores were consistent with the previous work from this lab demonstrates the reliability of the RGS to consistently predict pain during NTG migraine episodes. Although the findings were modest, treatment animals displayed higher levels of pain than control animals, suggesting the experience of migraine pain. There seemed to be a small effect for frequency of prior NTG migraine episodes, which could indicate that increased NTG exposure exacerbated migraine pain. These findings suggest that the rat grimace scale is reliable and a clinically valid tool for assessing pain in the NTG migraine model.

In humans, migraine pain is often exacerbated by physical activity (IHS, 2005). To assess this in our model, animals were placed in a modified light/dark box that automatically recorded movement. As we predicted, both 3 and 5 NTG migraine episodes decreased movement compared to animals receiving no NTG migraine episodes. This confirms the aggravation of migraine pain by movement in this model whish is also seen in the human clinical profile. Both 3 and 5 NTG exposures produced the same reduction in movement, suggesting that movement sensitivity is unrelated to the number of previous migraine episodes.

These results are in contrast to the previous studies from this lab, which showed that a single NTG migraine episode increased movement. We hypothesized that this was due to the novel experience of one NTG exposure and that increasing the number of migraine episodes to meet the clinical diagnostic criteria would reverse these findings. Multiple NTG migraine episodes did in fact reduce movement, presumably because animals had multiple opportunities to learn which behaviors alleviated or aggravated

migraine pain prior to test day. Multiple NTG migraine episodes reduced movement when a single NTG migraine episode increased it, indicating that multiple NTG migraine episodes are a more clinically relevant simulation of human migraine than a single NTG migraine episode. This is the first research, to our knowledge, that confirms movement sensitivity during an NTG migraine episode. Demonstrating the sensitivity to movement associated with multiple NTG administrations offers additional support for the validity of this model and the use of this assay as a dependent variable for ascertaining the presence of migraine.

Another common feature of migraine episodes is photophobia, or sensitivity to light (IHS, 2005). In this study, photophobia was assessed with the same modified light/dark box used to quantify movement. The 3 NTG group spent less time in the light chamber than the control group, and this difference approached significance. This trend was more robust in the 5 NTG group, which spent significantly less time in the light chamber than the control group, in accordance with our predictions.

The 3 NTG group spent less time in the light chamber than the control group but more time than the 5 NTG group. This "dose effect" of previous NTG migraine episodes could be caused by two factors. The first is that, like pain, sensitivity to light intensifies with subsequent migraine episodes. This suggests that some characteristics of NTG migraine episodes, such as pain and photophobia, worsen with increased NTG exposure, while other features of migraine, such as sensitivity to movement, remain stable once they have emerged. These "dose effects" are modest, but they do point to interesting topics for further study.

An alternative explanation for the "dose effect" seen in these results is a learning curve based on previous NTG exposure. Experiencing multiple migraine episodes in their home cage with the opaque enclosure allowed animals to learn that light exacerbated migraine pain. Avoiding light reduced treatment animals' level of pain, thus negatively reinforcing their photophobic behavior. Because the 5 NTG group experienced more migraine episodes than the 3 NTG group, the 5 NTG group may have developed a stronger association between light and pain. This would account for the more robust photophobia displayed in the 5 NTG group than the 3 NTG group.

These findings are in contrast to the previous study from this lab, which failed to show photophobia. This was most likely due to the novel experience of a single NTG migraine episode. By administering multiple migraine episodes and including the opaque covering, we showed a robust sensitivity to light that matches the clinical profile of human migraine. To our knowledge, this is the first evidence of photophobia in an NTG migraine model. Verifying the presence of photophobia associated with NTG administration provides additional support for this model of migraine and this assay as a valid assessment tool for photophobia.

Allodynia is a sensitivity to non-noxious stimuli that is sometimes seen in chronic migraineurs (Bigal et al., 2009). In the present study we found no evidence of thermal allodynia across treatment groups. These findings are consistent with and expand upon our previous work that failed to show thermal allodynia after one NTG administration. One reason this assay may have failed to reveal any significant results is that allodynia is almost exclusively seen in chronic migraineurs (Bigal et al., 2009). Our recurring model of migraine, with a maximum of 5 NTG migraine episodes, does not approach the attack

frequency of chronic migraine in humans: 15 headache days per month for a minimum of 3 months (IHS, 2005). From a clinical standpoint, we would not expect to see the presence of thermal allodynia after only 5 NTG migraine episodes.

These findings are in contrast to the Bates et al. study, however, which identified both thermal and mechanical allodynia in response to a single NTG exposure (Bates et al., 2010). Critical differences between the research from this lab and the Bates et al. study were the assessment measures and the species of animal used. The procedures employed by the Bates et al. study to assess allodynia were precise and well-established techniques, such as the Hargreaves and von Frey methods (Bates et al., 2010). Our lab did not have access to these technologies for rats, compelling us to use the thermal tail flick test. While the tail flick test is sound in theory, it is difficult to quantify reliably. Judging the difference between a tail flick and a reflex or other small movement was difficult and highly subjective, rendering this assay problematic for such a sensitive behavioral end point. The Bates et al. study also used mice, which are smaller and may be more susceptible to high doses of pharmacological compounds than rats (Bates et al., 2010). The lack of evidence for allodynia in this model may be explained by the deficiency of migraine exposures, the assessment method and/or the species of animal used. Further research may attempt to explore and correct these methodological issues.

Many migraineurs also exhibit comorbid anxiety and depressive disorders that become more common with increasing attack frequency (Zwart et al., 2003). In an attempt to quantify anxious and depressive symptoms in our model, animals were tested on the elevated plus maze and forced swim test. In contrast to our predictions, the results of these tests indicated no alterations in stress related behaviors on either test. Although

many migraineurs display psychiatric comorbidity, these disorders are more common under the chronic migraine condition in humans (Zwart et al., 2003). As previously discussed, our model of recurring migraine does not approach the attack frequency of chronic migraine. Although these results are somewhat disappointing, we may not expect to see anxiety and depressive symptoms after only 5 NTG migraine episodes. Clinically, one should not consider 5 NTG migraine episodes sufficient to model chronic migraine as they do not match in symptom frequency, expression of allodynia, or psychiatric comorbidity.

The recurring NTG migraine model yields two of the most clinically relevant behavioral endpoints for human migraine: sensitivity to movement and photophobia. Some assays have suggested additional homologies with human migraine including diminished weight gain, which we attributed to nausea, and pain expression in the rat grimace scale. Allodynia and comorbid disorders were not detected under the recurrent migraine model. This follows the clinical picture as allodynia and comorbid disorders are more commonly seen in the chronic migraine condition.

The validity of clinical simulations also requires sensitivity to pharmacotherapeutics intended to treat the condition. Two studies have found that sumatriptan, the gold standard anti-migraine treatment, reverses NTG-induced allodynia and hyperalgesia in mice (Bates et al., 2010; Pradhan et al., 2013). While these results support the NTG migraine model, the more clinically relevant endpoints determined in the present study must be reversed by sumatriptan to solidify the validity of this model. These studies are currently underway in this laboratory.

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APPENDIX

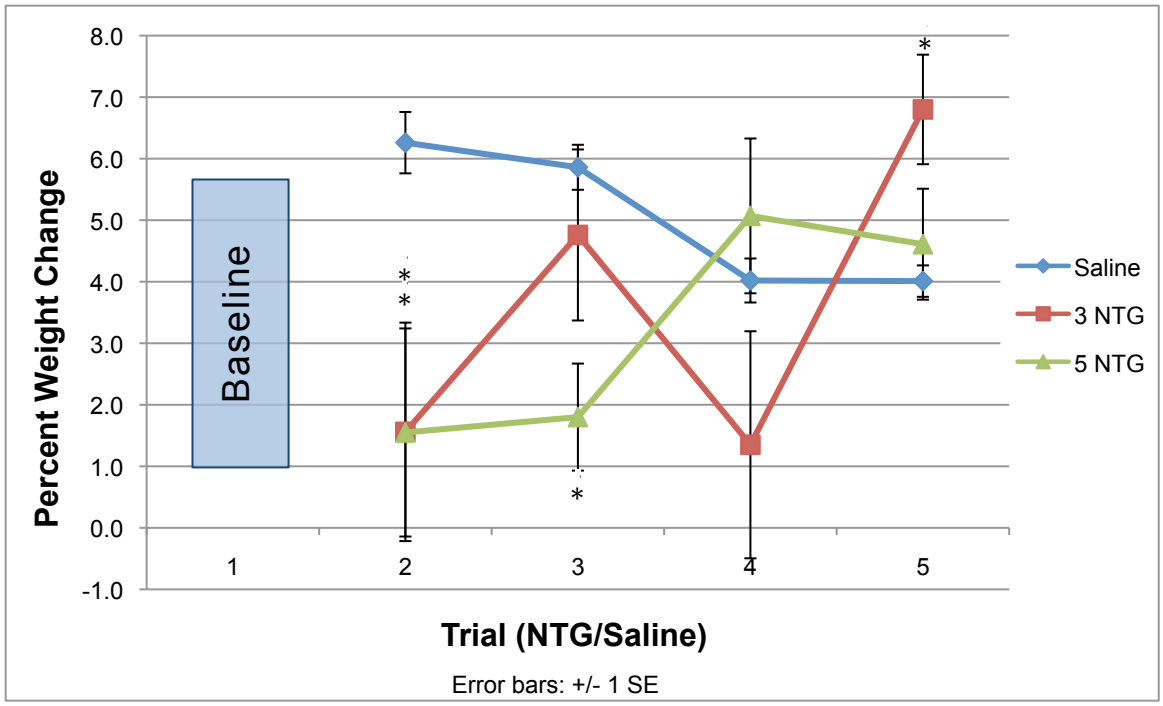

Figure 1A. Percent Weight Change by Trial Day. Data points represent group means +/- SEM, three days post trial days 1-4. No data is shown for trial day 1 because baseline weights were taken this day. The control group received saline on every trial day. The 3 NTG group received NTG on days 1 and 3 and saline on days 2 and 4. The 5 NTG group received NTG on every trial day. The highest percent weight change was seen post-saline administration and the lowest percent weight change was seen post-NTG administration. NTG administration decreased appetite compared to saline administration, suggesting the presence of nausea.

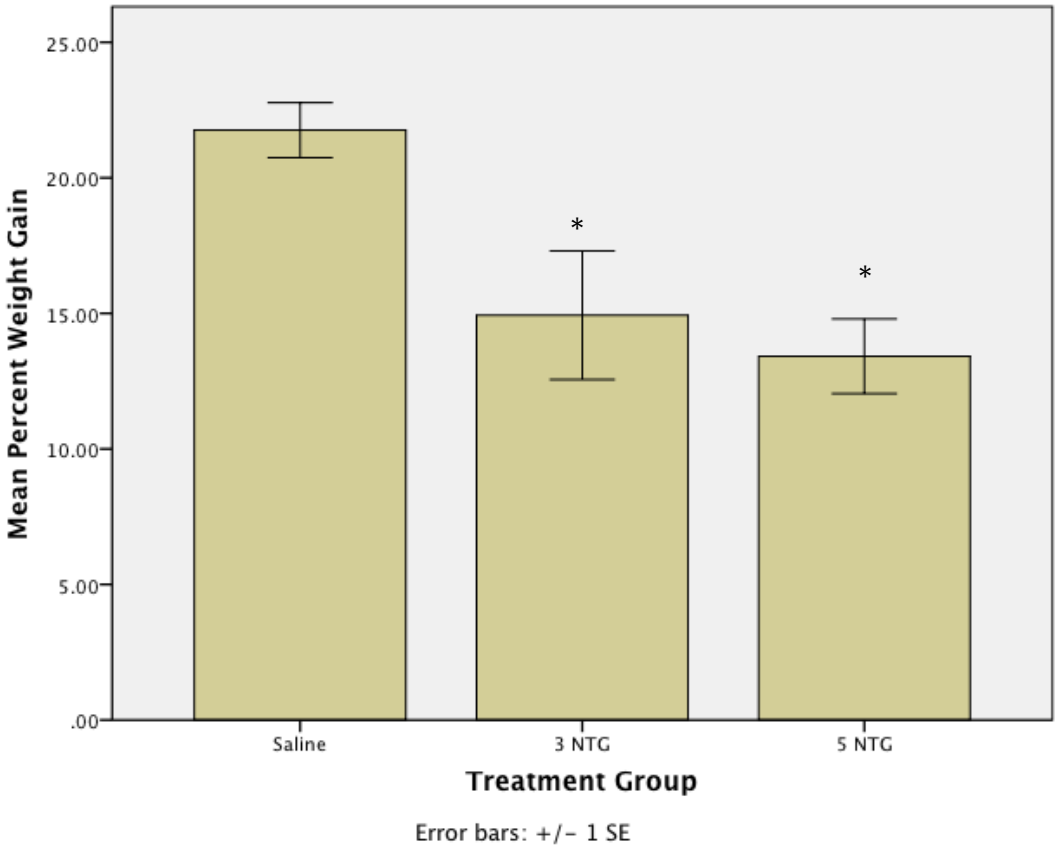

Figure 1B. Percent Weight Gain. Bars represent group means +/- SEM. Both 3 and 5 NTG administrations decreased percent weight gain over the two-week injection period compared to the control group (ps = .005 - .021).

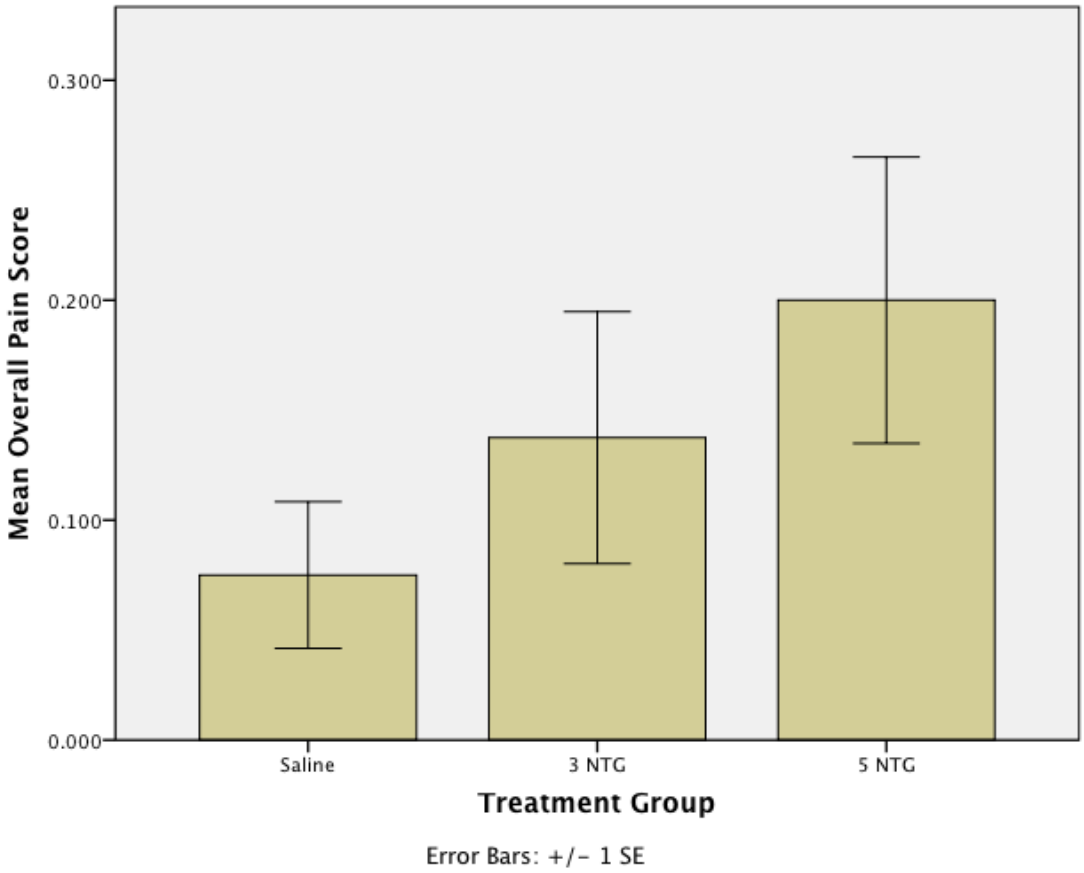

Figure 2. Rat Grimace Scale. Bars represent group means +/- SEM. No significant treatment effect was observed on rat grimace scores.

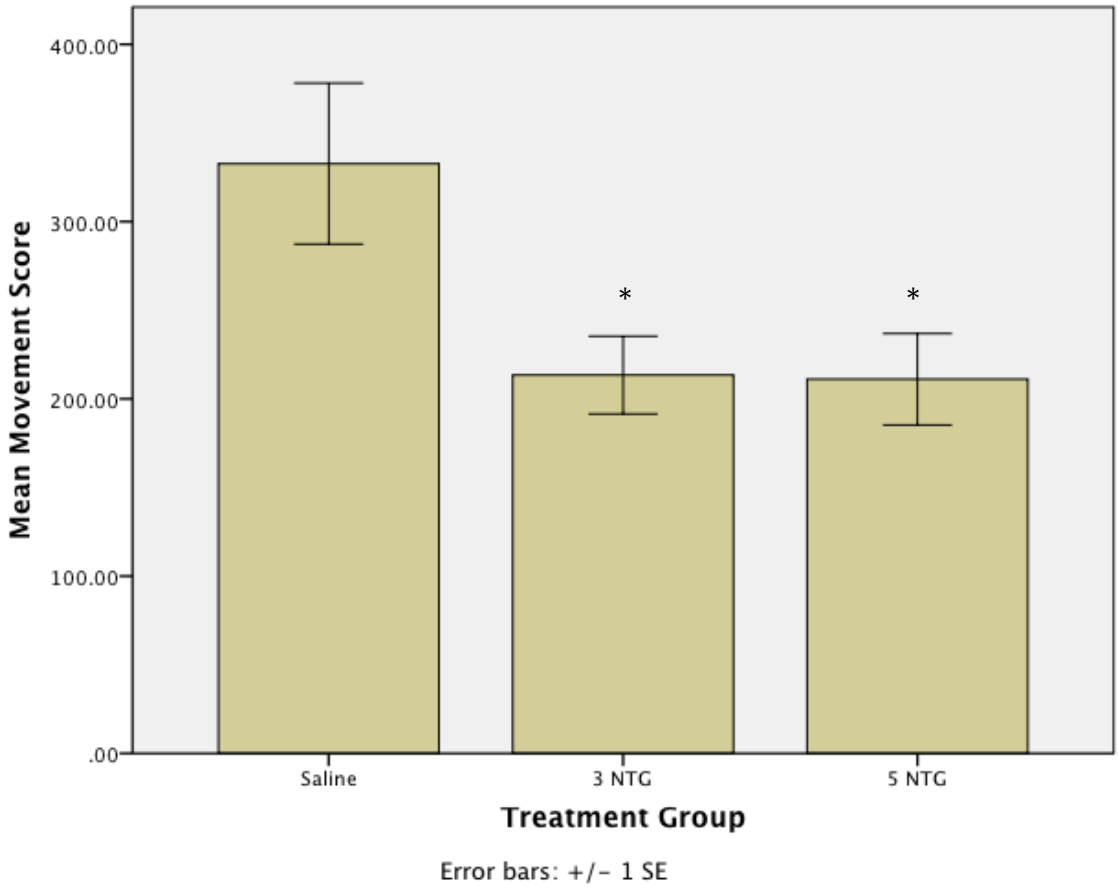

Figure 3. Movement in the Modified Light/dark Box. Bars represent group means +/- SEM. Both 3 and 5 NTG administrations decreased movement compared to the control group, indicating the presence of movement sensitivity (ps = .036 - .04).

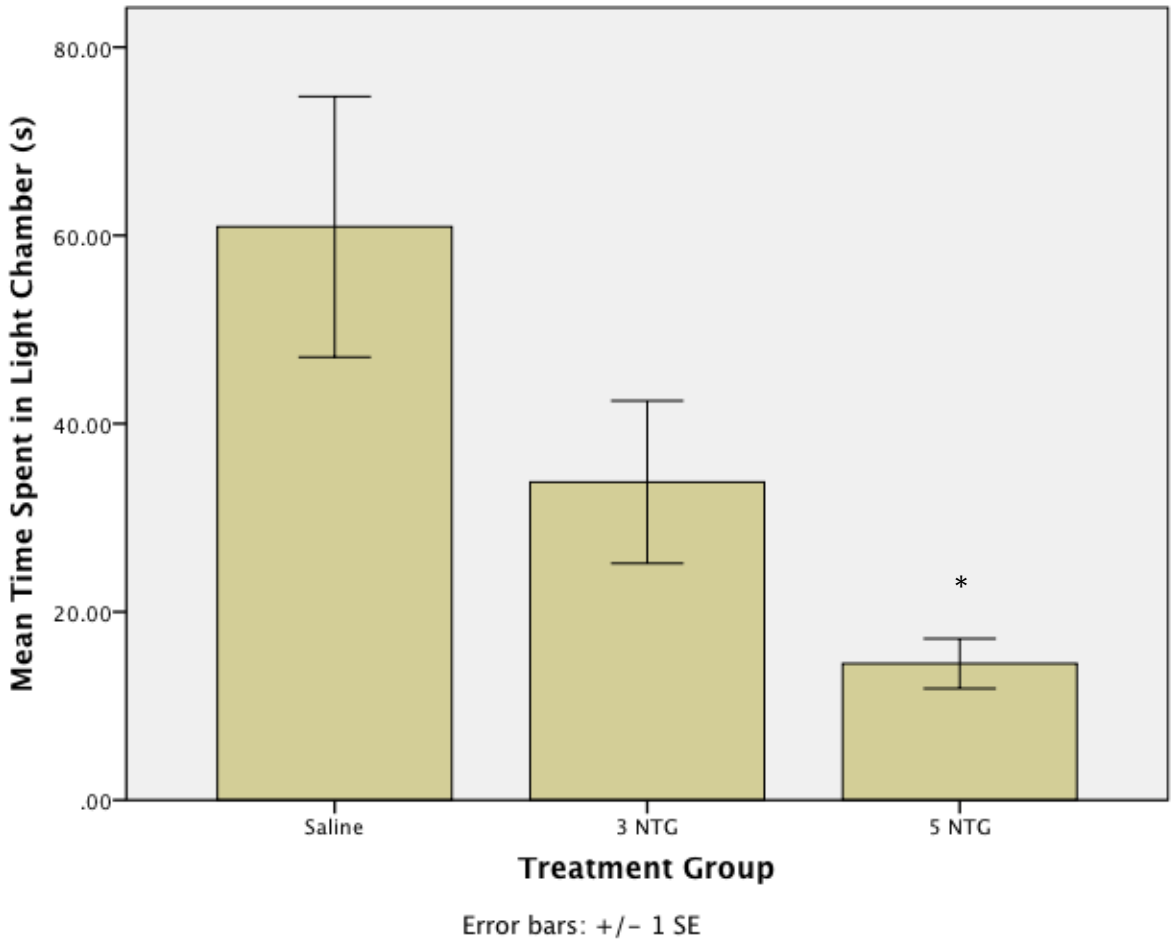

Figure 4. Time Spent in the Light Chamber of the Modified Light/dark Box. Bars represent group means +/- SEM. The reduction in the time spent in the light chamber of the three NTG group approached significance ($p = .055$). Five NTG administrations significantly decreased time spent in the light chamber, indicating the presence of photophobia ($p = .005$).

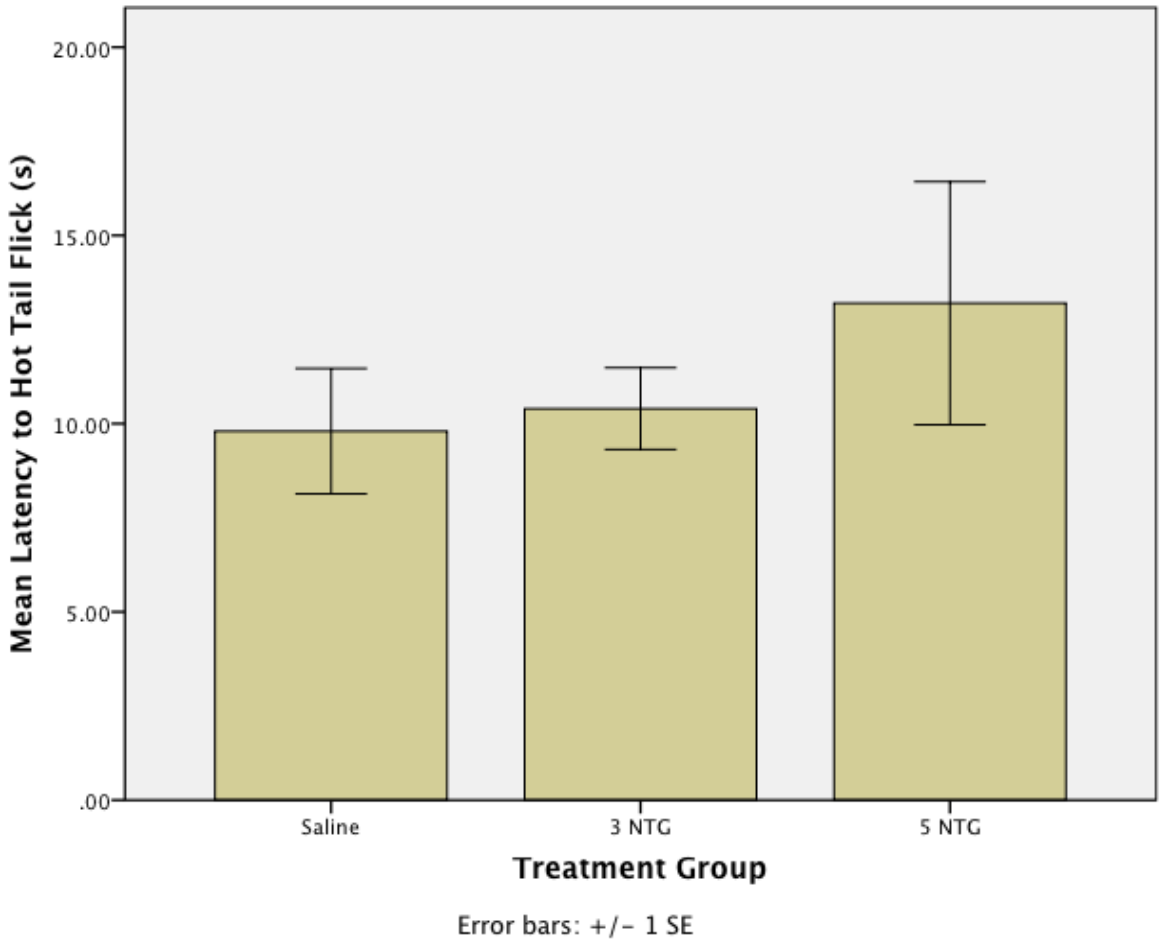

Figure 5. Hot Tail Flick Latency. Bars represent group means +/- SEM. No group differences were observed for hot tail flick latency.

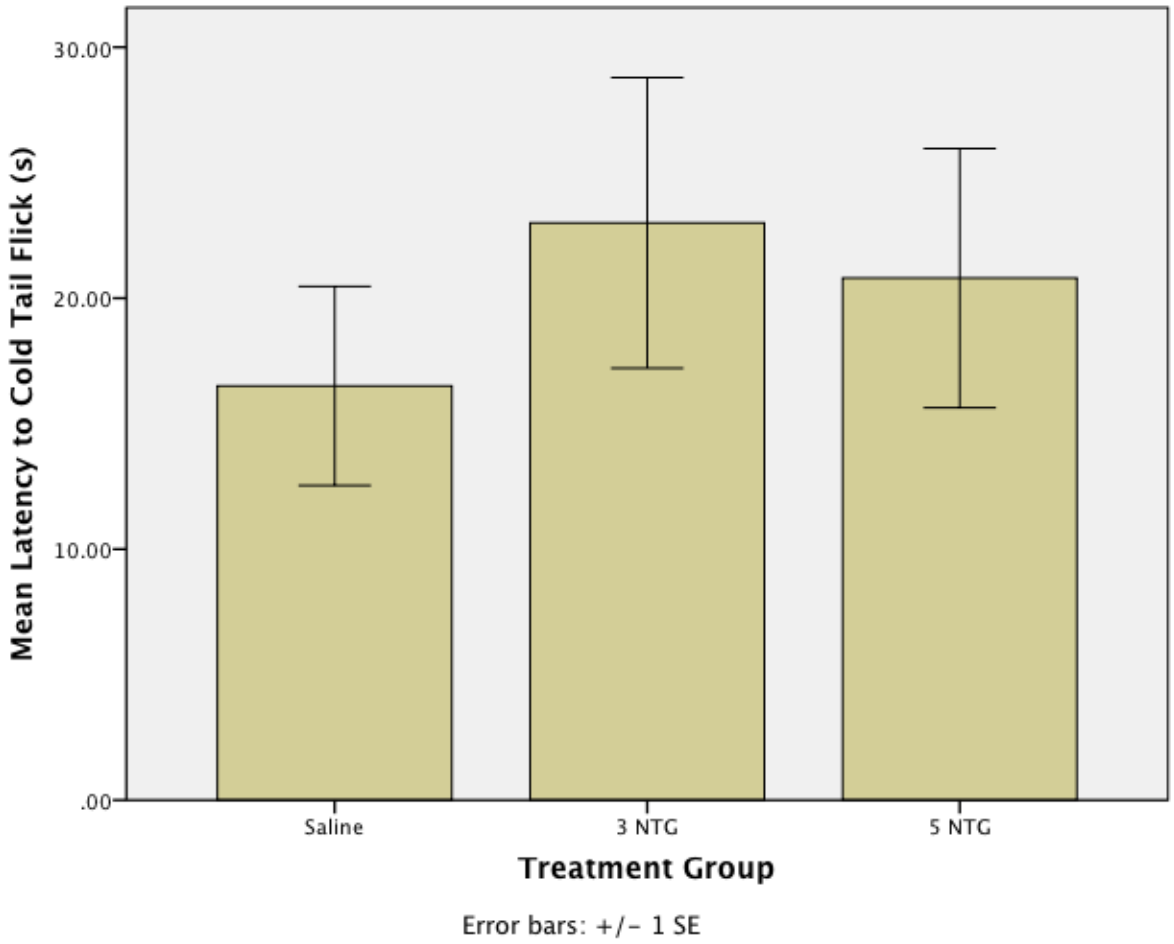

Figure 6. Cold Tail Flick Latency. Bars represent group means +/- SEM. No group differences were observed for cold tail flick latency.

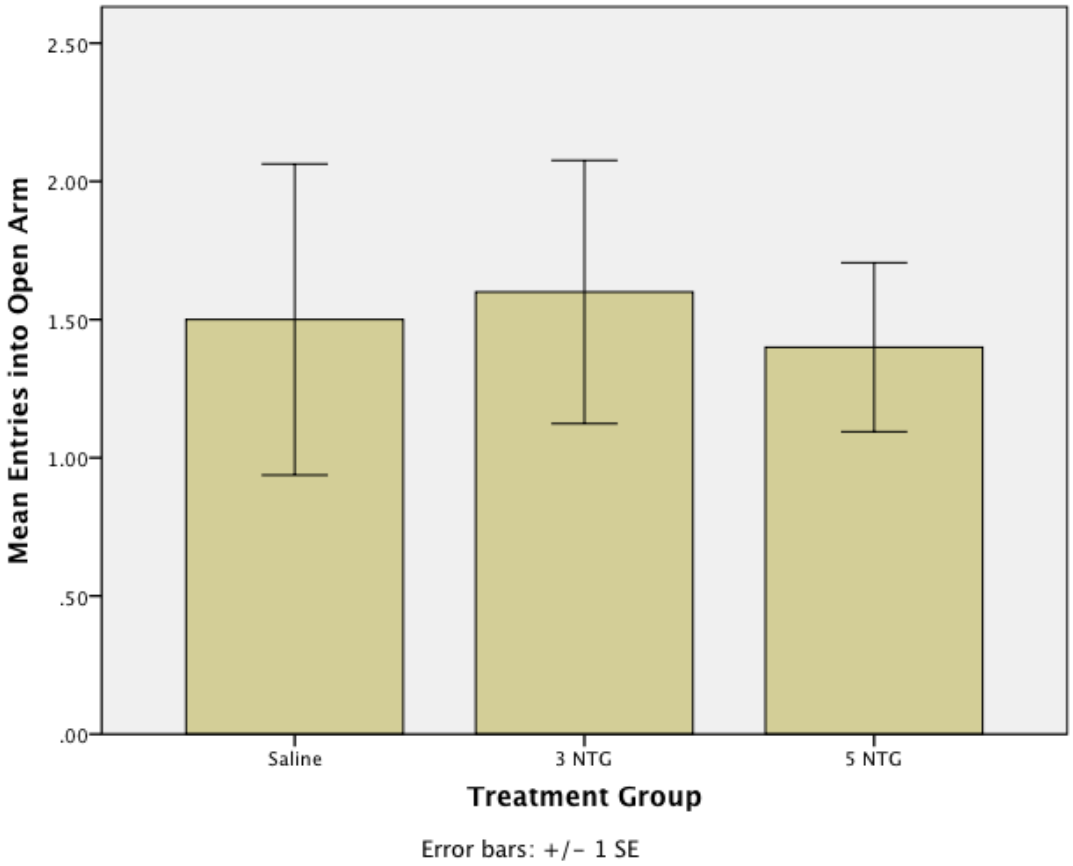

Figure 7. Open Arm Entries in the Elevated Plus Maze. Bars represent group means +/- SEM. No group differences were observed for open arm entries in the elevated plus maze.

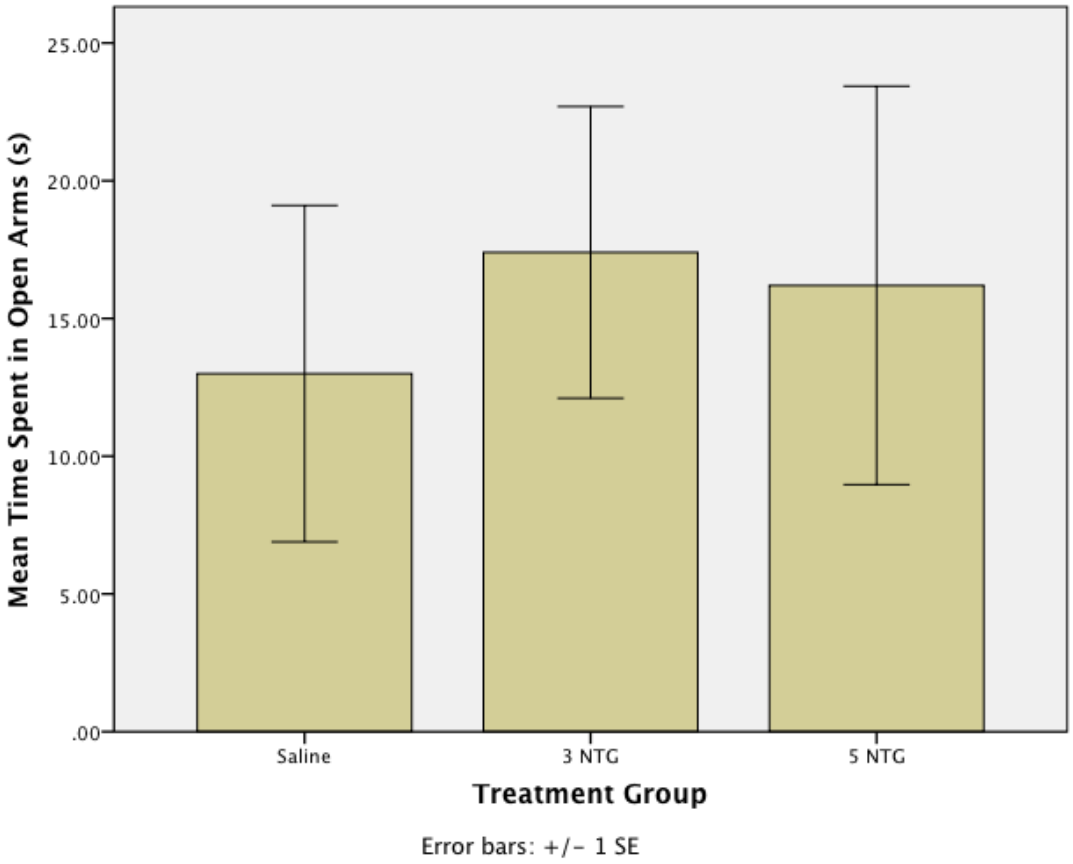

Figure 8. Time Spent in Open Arms of the Elevated Plus Maze. Bars represent group means +/- SEM. No group differences were observed for time spent in open arms of the elevated plus maze.

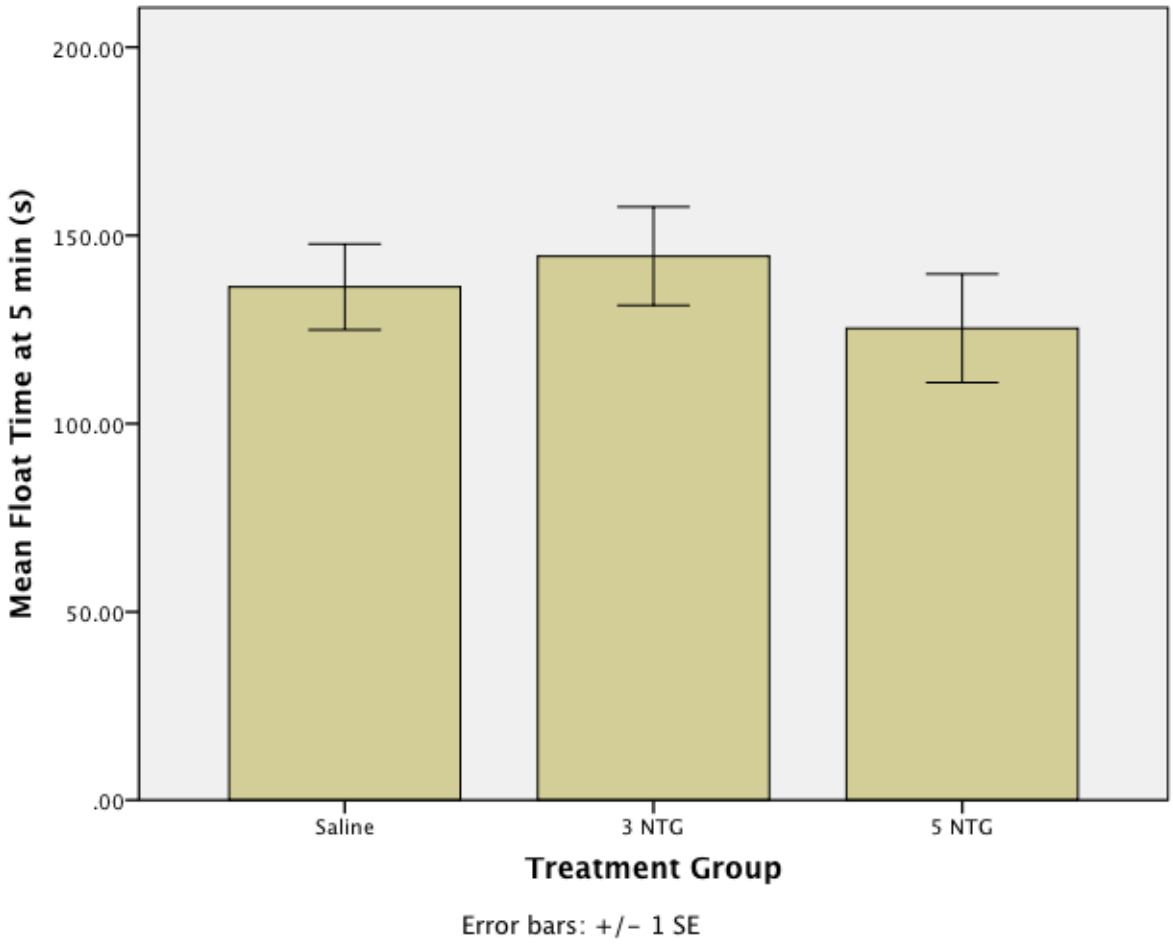

Figure 9. Float Time at 5 Minutes of the Forced Swim Test. Bars represent group means +/- SEM. No group differences were observed for float time at 5 minutes of the forced swim test.

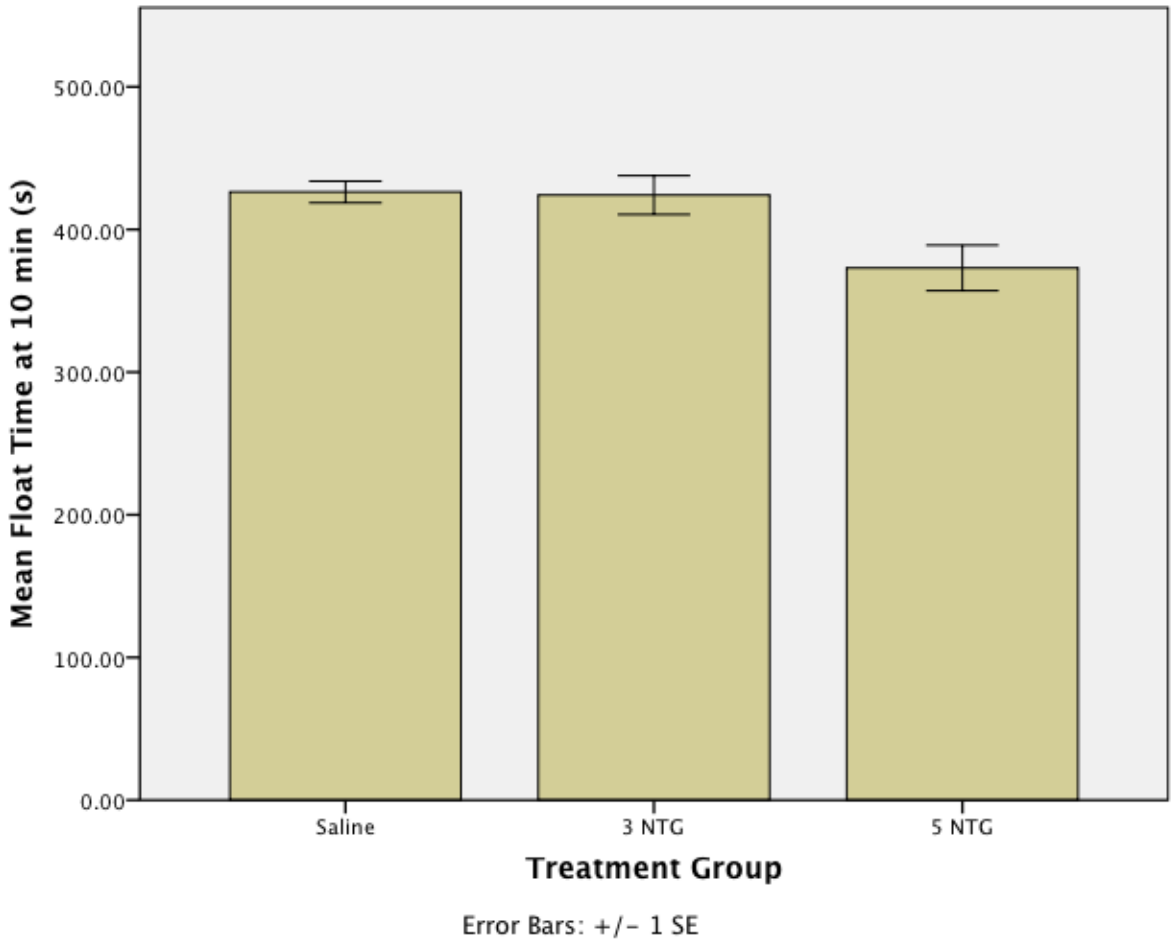

Figure 10. Float time at 10 Minutes of the Forced Swim Test. Bars represent group means +/- SEM. No group differences were observed for float time at 10 minutes of the forced swim test.